Application No. 10/612,497
Reply to March 15, 2005 Office Action
Reply Dated April 21, 2005

Amendments to the Specification:

Please replace the title on page 1, line 1 with the following title:

Methods For Producing Human Monoclonal Antibodies To CTLA-4

Please replace the paragraph from page 1, lines 8-11, with the following amended paragraph:

The present application is a divisional application of U.S. Patent Application 09/472,087, filed December 23, 1999, now U.S. Patent 6,682,736, which claims priority to U.S. Provisional Patent Application 60/113,647, filed December 23, 1998, the disclosures of which are hereby incorporated in their entirety herein.

Please replace the paragraphs from page 11 line 1 to page 12, line 12, with the following amended paragraphs:

Figure 2 provides a sequence alignment between the predicted heavy chain amino acid sequences from the clones 4.1.1 (SEQ ID NO: 74), 4.8.1 (SEQ ID NO: 75), 4.14.3 (SEQ ID NO: 78), 6.1.1 (SEQ ID NO: 79), 3.1.1 (SEQ ID NO: 73), 4.10.2 (SEQ ID NO: 76), 4.13.1 (SEQ ID NO: 77), 11.2.1 (SEQ ID NO: 80), 11.6.1 (SEQ ID NO: 81), 11.7.1 (SEQ ID NO: 82), 12.3.1.1 (SEQ ID NO: 83), and 12.9.1.1 (SEQ ID NO: 84) and the germline DP-50 (3-33) amino acid sequence (SEQ ID NO: 72). Differences between the DP-50 germline sequence and that of the

sequence in the clones are indicated in bold. The Figure also shows the positions of the CDR1, CDR2, and CDR3 sequences of the antibodies as shaded.

Figure 3 provides a sequence alignment between the predicted heavy chain amino acid sequence of the clone 2.1.3 (SEQ ID NO: 86) and the germline DP-65 (4-31) amino acid sequence (SEQ ID NO: 85). Differences between the DP-65 germline sequence and that of the sequence in the clone are indicated in bold. The Figure also shows the positions of the CDR1, CDR2, and CDR3 sequences of the antibody as underlined.

Figure 4 provides a sequence alignment between the predicted kappa light chain amino acid sequence of the clones 4.1.1 (SEQ ID NO: 88), 4.8.1 (SEQ ID NO: 89), 4.14.3 (SEQ ID NO: 90), 6.1.1 (SEQ ID NO: 91), 4.10.2 (SEQ ID NO: 92), and 4.13.1 (SEQ ID NO: 93) and the germline A27 amino acid sequence (SEQ ID NO: 87). Differences between the A27 germline sequence and that of the sequence in the clone are indicated in bold. The Figure also shows the positions of the CDR1, CDR2, and CDR3 sequences of the antibody as underlined. Apparent deletions in the CDR1s of clones 4.8.1, 4.14.3, and 6.1.1 are indicated with "0s".

Figure 5 provides a sequence alignment between the predicted kappa light chain amino acid sequence of the clones 3.1.1 (SEQ ID NO: 95), 11.2.1 (SEQ ID NO: 96), 11.6.1 (SEQ ID NO: 97), and 11.7.1 (SEQ ID NO: 98) and the germline 012 amino acid sequence (SEQ ID NO: 94). Differences between the 012 germline sequence and that of the sequence in the clone are

indicated in bold. The Figure also shows the positions of the CDR1, CDR2, and CDR3 sequences of the antibody as underlined.

Figure 6 provides a sequence alignment between the predicted kappa light chain amino acid sequence of the clone 2.1.3 (SEQ ID NO: 112) and the germline A10/A26 amino acid sequence (SEQ ID NO: 99). Differences between the A10/A26 germline sequence and that of the sequence in the clone are indicated in bold. The Figure also shows the positions of the CDR1, CDR2, and CDR3 sequences of the antibody as underlined.

Figure 7 provides a sequence alignment between the predicted kappa light chain amino acid sequence of the clone 12.3.1 (SEQ ID NO: 114) and the germline A17 amino acid sequence (SEQ ID NO: 113). Differences between the A17 germline sequence and that of the sequence in the clone are indicated in bold. The Figure also shows the positions of the CDR1, CDR2, and CDR3 sequences of the antibody as underlined.

Figure 8 provides a sequence alignment between the predicted kappa light chain amino acid sequence of the clone 12.9.1 (SEQ ID NO: 116) and the germline A3/A19 amino acid sequence (SEQ ID NO: 115). Differences between the A3/A19 germline sequence and that of the sequence in the clone are indicated in bold. The Figure also shows the positions of the CDR1, CDR2, and CDR3 sequences of the antibody as underlined.

Please replace the paragraph from page 30, lines 9-31, with the following amended paragraph:

Peptide analogs are commonly used in the pharmaceutical industry as non-peptide ~~drus~~ drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics". Fauchere, *J. Adv. Drug Res.* 15:29 (1986); Veber and Freidinger *TINS* p.392 (1985); and Evans et al. *J. Med. Chem.* 30:1229 (1987), which are incorporated herein by reference. Such compounds are often developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biochemical property or pharmacological activity), such as human antibody, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: --CH_2NH--, --CH_2S--, --CH_2-CH_2--, --CH=CH--(cis and trans), --$COCH_2$--, --$CH(OH)CH_2$--, and --CH_2SO--, by methods well known in the art. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Gierasch *Ann. Rev. Biochem.* 61:387 (1992), incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

Please replace the paragraph from page 36, lines 6-29, with the following amended paragraph:

This general strategy was demonstrated in connection with our generation of the first ~~XenoMouse~~XENOMOUSE® mouse strains as published in 1994. *See* Green et al. *Nature Genetics* 7:13-21 (1994). The ~~XenoMouse~~XENOMOUSE® mouse strains were engineered with yeast artificial chromosomes (YACs) containing 245 kb and 190 kb-sized germline configuration fragments of the human heavy chain locus and kappa light chain locus, respectively, which contained core variable and constant region sequences. *Id.* The human Ig containing YACs proved to be compatible with the mouse system for both rearrangement and expression of antibodies and were capable of substituting for the inactivated mouse Ig genes. This was demonstrated by their ability to induce B-cell development, to produce an adult-like human repertoire of fully human antibodies, and to generate antigen-specific human Mabs. These results also suggested that introduction of larger portions of the human Ig loci containing greater numbers of V genes, additional regulatory elements, and human Ig constant regions might recapitulate substantially the full repertoire that is characteristic of the human humoral response to infection and immunization. The work of Green et al. was recently extended to the introduction of greater than approximately 80% of the human antibody repertoire through introduction of megabase sized, germline configuration YAC fragments of the human heavy chain loci and kappa light chain loci, respectively, to produce ~~XenoMouse~~XENOMOUSE® mice. *See* Mendez et al. *Nature Genetics* 15:146-156 (1997), Green and Jakobovits *J. Exp. Med.* 188:483-495 (1998), and

U.S. Patent Application Serial No. 08/759,620, filed December 3, 1996, the disclosures of which are hereby incorporated by reference.

Please replace the paragraph from page 47, lines 1-2, with the following amended paragraph:

Further, combinatorial libraries can be designed and ~~sythesized~~ synthesized and used in screening programs, such as high throughput screening efforts.

Please replace the paragraph from page 47, lines 6-25, with the following amended paragraph:

It will be appreciated that administration of therapeutic entities in accordance with the invention will be administered with suitable carriers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15^{th} ed, Mack Publishing Company, Easton, PA (1975)), particularly Chapter 87 by Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as ~~Lipofectin~~LIPOFECTIN™ vesicles), DNA conjugates, anhydrous

absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in treatments and therapies in accordance with the present invention, provided that the active ingredient in the formulation is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. *See also* Powell et al. "Compendium of excipients for parenteral formulations" *PDA J Pharm Sci Technol.* 52:238-311 (1998) and the citations therein for additional information related to excipients and carriers well known to pharmaceutical chemists.

Please replace the paragraph from page 48, lines 10-22, with the following amended paragraph:

Through use of such technology, we have produced fully human monoclonal antibodies to a variety of antigens. Essentially, we immunize ~~XenoMouse~~XENOMOUSE® lines of mice with an antigen of interest, recover lymphatic cells (such as B-cells) from the mice that express antibodies, fuse such recovered cells with a myeloid-type cell line to prepare immortal hybridoma cell lines, and such hybridoma cell lines are screened and selected to identify hybridoma cell lines that produce antibodies specific to the antigen of interest. We utilized these techniques in accordance with the present invention for the preparation of antibodies specific to CTLA-4. Herein, we describe the production of multiple hybridoma cell lines that produce

antibodies specific to CTLA-4. Further, we provide a characterization of the antibodies produced by such cell lines, including nucleotide and amino acid sequence analyses of the heavy and light chains of such antibodies.

Please replace the paragraph from page 49, line 29 to page 50, line 5, with the following amended paragraph:

Further, expression of antibodies of the invention (or other moieties therefrom) from production cell lines can be enhanced using a number of known techniques. For example, the glutamine ~~sythetase~~ synthetase and DHFR gene expression systems are common approaches for enhancing expression under certain conditions. High expressing cell clones can be identified using conventional techniques, such as limited dilution cloning and Microdrop technology. The GS system is discussed in whole or part in connection with European Patent Nos. 0 216 846, 0 256 055, and 0 323 997 and European Patent Application No. 89303964.4.

Please replace the paragraph from page 56, line 30 to page 57, line 2, with the following amended paragraph:

Antigen Preparation: Three distinct immunogens were prepared for immunization of the ~~XenoMouse~~XENOMOUSE® mice: (i) a CTLA-4-IgG fusion protein, (ii) a

CTLA-4 peptide, and (iii) 300.19 murine lymphoma cells transfected with a mutant of CTLA-4 (Y201V) that is constitutively expressed on the cell surface.

Please replace on page 57, line 21, with the following amended line:

OM-CTLA4-IgGl Fusion Protein (SEQ ID NO: 100).

Please replace the paragraph page 59, line 25 to page 60, line 4 with the following amended paragraph:

The purified CTLA-4-IgGl migrated as a single band on SDS-PAGE using colloidal coomassie staining (Novex). Under non-reducing conditions CTLA-4-IgGl was a dimer (100kDa), that reduced to a 50kDa monomer when treated with 50mM DTT. Amino acid sequencing of the purified CTLA-4-IgG1 in solution confirmed the N-terminus of CTLA-4 (MHVAQPAVVLAS) (SEQ ID NO: 101), and that the oncostatin-M signal peptide was cleaved from the mature fusion protein. The CTLA-4-IgGl bound to immobilized B7.1-IgG in a concentration dependent manner and the binding was blocked by a hamster-anti-human anti-CTLA-4 antibody (BNI3: PharMingen). The sterile CTLA-4-IgG was endotoxin free and quantitated by OD280 using 1.4 as the extinction coefficient. The yield of purified CTLA-4-IgG ranged between 0.5-3mgs/liter of CHO-K1 cells.

Please replace page 60, line 8, with the following amended line:

The following CTLA-4 peptide (SEQ ID NO: 102) was prepared as described

Please replace the paragraph from page 61, lines 10-28, with the following amended paragraph:

25mg aliquots of the crude CTLA-4 peptide were dissolved in 5ml 6M Guanidine HCl/100mM K_2PO_3 at pH6.4 and eluted over a Pharmacia ~~Hi-Load Superdex~~ HILOAD™ SUPERDEX™ 75 16/60 column (16mm x 600mm, 120ml bed volume) with 2M Guanidine.HCl / 100mM K_2PO_3 at pH6.4 at 2 ml / min for 180 minutes collecting 5 ml fractions. The fractions were analyzed by loading 1.7μl of fractions onto a NuPAGE Laemeli gel running with MES running buffer and visualizing via Daichii silver stain protocol. Those fractions exhibiting a molecular weight of 12 KDa, as judged versus molecular weight standards, were pooled together and stored at 4°C. The combined fractions were analyzed by UV and gel electrophoresis. Amino acid sequencing was performed by absorbing a 100 microliter sample in a ~~ProSorb~~ PROSORB® cartridge (absorbed onto a PVDF membrane) and washing to remove the buffer salts. Sequencing was performed on an Applied Biosystems 420. The expected N-terminal sequence (M H V A Q P A V V L A) (SEQ ID NO: 103) was observed. Immunoblotting demonstrated that the peptide was recognized by the BNI3 anti-human CTLA-4 (PharMingen). To desalt, an aliquot containing 648μg of material was placed in 3500 Da MWCO dialysis tubing and dialyzed against 0.1% TFA / H2O at 4°C for 9 days with stirring. The entire contents of the dialysis bag was ~~lyophilyzed~~ lyophilized to a powder.

Please replace the paragraph from page 62, line 21 to page 63, line 3, with the following amended paragraph:

Immunization and hybridoma generation: ~~XenoMouse~~XENOMOUSE® mice (8 to 10 weeks old) were immunized (i) subcutaneously at the base of tails with 1×10^7 300.19 cells that were transfected to express CTLA-4 as described above, resuspended in phosphate buffered saline (PBS) with complete Freund's adjuvant, or (ii) subcutaneously at the base of tail with (a) 10 μg the CTLA-4 fusion protein or (b) 10 μg CTLA-4 peptide, emulsified with complete Freund's adjuvant. In each case, the dose was repeated three or four times in incomplete Freund's adjuvant. Four days before fusion, the mice received a final injection of the immunogen or cells in PBS. Spleen and/or lymph node lymphocytes from immunized mice were fused with the [murine non-secretory myeloma P3 cell line] and were subjected to HAT selection as previously described (Galfre, G. and Milstein, C., "Preparation of monoclonal antibodies: strategies and procedures." *Methods Enzymol.* 73:3-46 (1981)). A large panel of hybridomas all secreting CTLA-4 specific human $IgG_2\kappa$ or $IgG_4\kappa$ (as detected below) antibodies were recovered.

Please replace the paragraph from page 64, lines 10-21, with the following amended paragraph:

Kinetic analysis of the antibodies was carried out using antigens immobilized onto the sensor surface at a low density. Three surfaces of the BIAcore sensorchip were ~~immobililized~~

immobilized with the CTLA-4-Ig fusion protein at a density ranging from approximately 390-900 using CTLA-4-Ig fusion protein at 20 or 50 μg/ml in 10 mM sodium acetate at pH 5.0 using the amine coupling kit supplied by the manufacturer (BIAcore, Inc.). The fourth surface of the BIAcore sensorchip was immobilized with IgG1 (900 RU) and was used as a negative control surface for non-specific binding. Kinetic analysis was performed at a flow rate of 25 or 50 microliters per minute and dissociation (kd or k_{off}) and association (ka or k_{on}) rates were determined using the software provided by the manufacturer (BIA evaluation 3.0) that allows for global fitting calculations.

Please replace the paragraph from page 66, line 15 to page 67, line 2, with the following amended paragraph:

Poly(A)$^+$ mRNA was isolated from approximately 2 X 10^5 hybridoma cells derived from immunized ~~XenoMouse~~XENOMOUSE® mice using a Fast-Track kit (Invitrogen). The generation of random primed cDNA was followed by PCR. Human V_H or human V_κ family specific variable region primers (Marks et al., "Oligonucleotide primers for polymerase chain reaction amplification of human immunoglobulin variable genes and design of family-specific oligonucleotide probes." *Eur. J. Immunol.* 21:985-991 (1991)) or a universal human V_H primer, MG-30 (CAGGTGCAGCTGGAGCAGTCIGG) (SEQ ID NO: 104) was used in conjunction with primers specific for the human Cγ2 constant region (MG-40d;

5'-GCTGAGGGAGTAGAGTCCTGAGGA-3') (SEQ ID NO: 105) or Cκ constant region (hκP2; as previously described in Green et al., 1994). Sequences of human Mabs-derived heavy and kappa chain transcripts from hybridomas were obtained by direct sequencing of PCR products generated from poly(A^+) RNA using the primers described above. PCR products were also cloned into pCRII using a TA cloning kit (Invitrogen) and both strands were sequenced using Prism dye-terminator sequencing kits and an ABI 377 sequencing machine. All sequences were analyzed by alignments to the "V BASE sequence directory" (Tomlinson et al., MRC Centre for Protein Engineering, Cambridge, UK) using MacVector and Geneworks software programs.

Please replace the paragraph from page 67, line 3 to page 68, line 7, with the following amended paragraph:

Further, each of the antibodies 4.1.1, 4.8.1, 11.2.1, and 6.1.1 were subjected to full length DNA sequences. For such sequencing, Poly$(A)^+$ mRNA was isolated from approximately 4 X 10^6 hybridoma cells using mRNA Direct kit (Dynal). The mRNA was reverse transcribed using oligo-dT(18) and the Advantage RT/PCR kit (Clonetech). The Variable region database (V Base) was used to design amplification primers beginning at the ATG start site of the heavy chain DP50 gene (5'-TATCTAAGCTTCTAGACTCGACCGCCACCATGGAGTTTGGGCTGAGCTG-3') (SEQ ID NO: 106) and to the stop codon of the IgG2 constant region (5'-

TTCTCTGATCAGAATTCCTATCATTTACCCGGAGACAGGGAGAGCT-3') <u>(SEQ ID NO: 107)</u>. An optimal Kozak sequence (ACCGCCACC) <u>(SEQ ID NO: 108)</u> was added 5' to the ATG start site. The same method was used to design a primer to the ATG start site of the kappa chain A27 gene (5'-

TCTTCAAGCTTGCCCGGGCCCGCCACC<u>ATG</u>GAAACCCCAGCGCAG

-3') <u>(SEQ ID NO: 109)</u> and the stop codon of the kappa constant region (5'-TTCTTTGATCAGAATTCTCACTAACACTCTCCCCTGTTGAAGC-3') <u>(SEQ ID NO: 110)</u>. The 012 cDNA was cloned by using a primer to the ATG start site (5'-TCTTCAAGCTTGCCCGGGCCCGCCACCATGGACATGAGGGTCCCCGCT-3) <u>(SEQ ID NO: 111)</u> and the kappa constant region stop codon primer above. The heavy chain cDNAs were also cloned as genomic constructs by site directed mutagenesis to add an NheI site at the end of the variable J domain and subcloning an NheI-fragment containing the genomic IgG2 CH1/Hinge/CH2/CH3 regions. The point mutation to generate NheI site does not alter the amino acid sequence from germline. The primer pairs were used to amplify the cDNAs using Advantage High Fidelity PCR Kit (Clonetech). Sequence of the PCR was obtained by direct sequencing using dye-terminator sequencing kits and an ABI sequencing machine. The PCR product was cloned into pEE glutamine synthetase mammalian expression vectors (Lonza) and three clones were sequenced to confirm somatic mutations. For each clone, the sequence was verified on both strands in at least three reactions. An aglycosylated 4.1.1 antibody was generated by site directed mutagenesis of N294Q in the CH2 domain. Recombinant

antibodies were produced by transient ~~trasnfection~~ transfection of Cos7 cells in IgG depleted FCS and purified using standard Protein A sepharose techniques. Stable transfectants were generated by electroporation of murine NSO cells and selection in glutamine free media. Recombinant 4.1.1 with or without glycosylation exhibited identical specificity and affinity for CTLA4 in the *in vitro* ELISA and BIAcore assays.

Please replace the paragraph from page 69, line 6 to page 70, line 4, with the following amended paragraph:

As will be observed, antibodies in accordance with the present invention were generated with a strong bias towards the utilization of the DP-50 heavy chain variable region. The DP-50 gene is also referred to as a V_H 3-33 family gene. Only one antibody that was selected on the basis of CTLA-4 binding and preliminary *in vitro* functional assays showed a heavy chain gene utilization other than DP-50. That clone, 2.1.3, utilizes a DP-65 heavy chain variable region and is an IgG4 isotype. The DP-65 gene is also referred to as a V_H 4-31 family gene. On the other hand, the clone, 4.9.1, which possesses a DP-47 heavy chain variable region binds to CTLA-4 but does not inhibit binding to B7-1 or B7-2. In ~~XenoMouse~~XENOMOUSE® mice, there are more than 30 distinct functional heavy chain variable genes with which to generate antibodies. Bias, therefore, is indicative of a preferred

binding motif of the antibody-antigen interaction with respect to the combined properties of binding to the antigen and functional activity.

Please replace the paragraph from page 70, lines 6-17, with the following amended paragraph:

As will be appreciated, gene utilization analysis provides only a limited overview of antibody structure. As the B-cells in ~~XenoMouse~~XENOMOUSE® animals ~~stocastically~~ stochastically generate V-D-J heavy or V-J kappa light chain transcripts, there are a number of secondary processes that occur, including, without limitation, somatic hypermutation, n-additions, and CDR3 extensions. *See, for example,* Mendez et al. *Nature Genetics* 15:146-156 (1997) and U.S. Patent Application Serial No. 08/759,620, filed December 3, 1996. Accordingly, to further examine antibody structure predicted amino acid sequences of the antibodies were generated from the cDNAs obtained from the clones. In addition, N-terminal amino acid sequences were obtained through protein sequencing.

Please replace the paragraph from page 73, lines 1-5, with the following amended paragraph:

Signal peptide sequences are shown in bold and large text. The open reading frames in the full length 4.1.1 genomic DNA sequence (Fig. 22(b)) are underlined. And, the mutations introduced to make the aglycosylated 4.1.1 heavy chain and the resulting change (N294Q) are shown in ~~doble~~ double underline and bold text (cDNA (Fig. 22(b) and amino acid (Fig. 22(c)).

Please replace the paragraph from page 75, lines 18-24, with the following amended paragraph:

SDS-PAGE was performed using the Novex NuPAGE electrophoresis system with a 10% NuPAGE gel and MES running buffer. Samples were prepared by diluting 3:1 with 4x NuPAGE sample buffer (+/-) beta-mercaptoethanol, heated and ~ 5 ~~u~~µg of protein was loaded onto the gel. The gel was then stained with Brilliant Blue R staining solution (Sigma cat.# B-6529) and molecular size estimates were made by comparing stained bands to ~~"Perfect Protein~~ PERFECT PROTEIN™ ~~M~~markers~~"~~ (Novagen cat# 69149-3).

Please replace the paragraph from page 76, lines 1-5, with the following amended paragraph:

Isoelectric focusing (IEF) was performed using Pharmacia IEF 3-9 pHast gels (cat# 17-0543-01) . Samples were diluted in 10% glycerol to ~0.8 mg/ml and 1 ~~u~~µl was loaded onto gel and then silver stained. The pI estimates were made by comparing stained bands to broad range (pH3-10) IEF standards (Pharmacia cat # 17-0471-01)

Please replace the paragraph from page 76, lines 12-20, with the following amended paragraph:

For FACS studies, human peripheral T cells were prepared and stimulated for 48 hours. T cells were washed once, resuspended in FACS buffer at 1×10^6 cells/100 ~~u~~µl and stained for CD3 surface expression with 10 ~~u~~µl of anti-CD3-FITC (Immunotech, Marseille, France) for 30 minutes at room temperature. Cells were washed twice, then fixed, permeabilized (Fix and Perm, Caltag), and stained for intracellular CTLA-4 expression with 10 ul anti-CD152-PE (Pharmingen). Flow cytometry was performed using a Becton Dickinson FACSort. Quadrants were set by analysis of relevant isotype control antibodies (Caltag).

Please replace the paragraph from page 77, line 18 to page 78, line 3, with the following amended paragraph:

3 nM B7.1-Ig(G1) or B7.2-Ig(G1) (Repligen, Inc. Needham, MA) in Dulbecco's PBS was coated on 96-well ~~MaxiSorp~~MAXISORP™ plates (Nunc, Denmark, #439454) and incubated at 4°C overnight. On day 2, B7-Ig was removed and plates were blocked with 1% BSA plus 0.05% ~~Tween~~TWEEN®-20 in D-PBS for two hours. Plates were washed 3X with wash buffer (0.05% ~~Tween~~TWEEN®-20 in D-PBS). Antibody at appropriate test concentrations and CTLA-4-Ig(G4) (0.3 nM final conc.) (Repligen, Inc. Needham, MA) were pre-mixed for 15 minutes and then added to the B7-Ig coated plate (60 ~~u~~µl total volume) and incubated at RT for 1.5 hours. Plates were washed 3X and 50 µl of a 1 to 1000 dilution of HRP-labeled mouse anti-human IgG4 antibody (Zymed, San Francisco, CA, #05-3820) was added and incubated at RT for 1 hour. Plates were washed 3X and 50 µl TMB Microwell peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD, #50-76-04) was added and incubated at RT for 20 minutes, and then 50 µl 1N H_2SO_4 was added to the plate. Plates were read at 450 nm using a Molecular Devices plate reader (Sunnyvale, CA). All samples were tested in duplicate. Maximal signal was defined as CTLA-4-Ig binding in the absence of test antibody. Non-specific binding was defined as absorbance in the absence of CTLA-4-Ig and test antibody.

Please replace the paragraph from page 79, lines 12-31, with the following amended paragraph:

A 96-well FluroNUNC plate (Nunc Cat No.475515) was platecoated with four antigens: CTLA-4/Ig, CD44/Ig, CD28/Ig, and B7.2/Ig (antigens generated in-house). The antigens were platecoated overnight at +4°C at 1~~u~~µg/ml 100~~u~~µl/well in 0.1M sodium bicarbonate buffer, pH 9.6. The plate was then washed with PBST (PBS + 0.1% ~~Tween~~TWEEN®-20) three times using a NUNC plate washer. The plate was blocked with PBST+0.5%BSA at 150 ~~u~~µl/well. The plate was incubated at RT for 1 hour then washed with PBST three times. Next the anti-CTLA-4 antibodies of the invention were diluted in block at 1 µg/ml and were added to the plate. The plate was incubated at RT for 1 hour then washed with PBST three times. The wells that contained the antibodies of the invention were then treated with 100 µl/well anti-human IgG2-HRP (Southern Biotech Cat No.9070-05) at a 1:4000 dilution in block. Also, one row was treated with anti-human IgG (Jackson Cat No. 209-035-088) to normalize for platecoating. This antibody was diluted to 1:5000 in block and added at 100 ~~u~~µl/well. Also, one row was treated with anti-human CTLA-4-HRP (Pharmingen Cat No. 345815/Custom HRP conjugated) as a positive control. This antibody was used at 0.05 ~~u~~µg/ml diluted in block. The plate was incubated at RT for 1 hour then washed with PBST three times. LBA chemiluminescent substrate (Pierce) was added at 100 µl/well and the plate was incubated on a plateshaker for 5 min. The plate was then read using a lumi-imager for a 2 min. exposure.

Please replace the paragraph from page 84, lines 21-24, with the following amended paragraph:

We developed a second cellular assay in order to quantify the enhancement of T-cell IL-2 upregulation upon blockade of CTLA-4 signal with the antibodies. The following materials and methods were utilized in connection with the ~~expeiments~~ experiments:

Please replace the paragraph from page 88, lines 24-25, with the following amended paragraph:

Coligan et al., Unit 2.1, "Enzyme-linked immunosorbent assays," in *Current ~~p~~Protocols in ~~irnmunology~~ Immunology* (1994)

Please replace the paragraph from page 92, lines 16-18, with the following amended paragraph:

Taylor et al., "Human immunoglobulin transgenes undergo rearrangement, somatic mutation and class switching in mice that lack endogenous IgM." *International ~~Irnmunology~~ Immunology* 6:579-591 (1994)